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# Mutagenesis of the Conserved Residue Glu$^{259}$ of $G_s\alpha$ Demonstrates the Importance of Interactions between Switches 2 and 3 for Activation*

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**We previously reported that substitution of Arg$^{258}$ within the switch 3 region of $G_s\alpha$ impaired activation and increased basal GDP release due to loss of an interaction between the helical and GTPase domains (Warner, D. R., Weng, G., Yu, S., Matalon, R., and Weinstein, L. S. (1998) *J Biol. Chem.* 273, 23976–23983). The adjacent residue (Glu$^{259}$) is strictly conserved in G protein $\alpha$-subunits and is predicted to be important in activation. To determine the importance of Glu$^{259}$, this residue was mutated to Ala ($G_s\alpha$-E259A), Gln ($G_s\alpha$-E259Q), Asp ($G_s\alpha$-E259D), or Val ($G_s\alpha$-E259V), and the properties of *in vitro* translation products were examined. The $G_s\alpha$-E259V was studied because this mutation was identified in a patient with Albright hereditary osteodystrophy. S49 cyc reconstitution assays demonstrated that $G_s\alpha$-E259D stimulated adenylyl cyclase normally in the presence of GTP$\gamma$S but was less efficient with isoproterenol or $AlF_4^-$. The other mutants had more severely impaired effector activation, particularly in response to $AlF_4^-$. In trypsin protection assays, GTP$\gamma$S was a more effective activator than $AlF_4^-$ for all mutants, with $G_s\alpha$-E259D being the least severely impaired. For $G_s\alpha$-E259D, the $AlF_4^-$-induced activation defect was more pronounced at low $Mg^{2+}$ concentrations. $G_s\alpha$-E259D and $G_s\alpha$-E259A purified from *Escherichia coli* had normal rates of GDP release (as assessed by the rate GTP$\gamma$S binding). However, for both mutants, the ability of $AlF_4^-$ to decrease the rate of GTP$\gamma$S binding was impaired, suggesting that they bound $AlF_4^-$ more poorly. GTP$\gamma$S bound to purified $G_s\alpha$-E259D irreversibly in the presence of 1 mM free $Mg^{2+}$, but dissociated readily at micromolar concentrations. Sucrose density gradient analysis of *in vitro* translates demonstrated that all mutants except $G_s\alpha$-E259V bind to $\beta\gamma$ at 0 °C and were stable at higher temperatures. In the active conformation Glu$^{259}$ interacts with conserved residues in the switch 2 region that are important in maintaining both the active state and $AlF_4^-$ in the guanine nucleotide binding pocket. Although both $G_s\alpha$ Arg$^{258}$ and Glu$^{259}$ are critical for activation, the mechanisms by which these residues affect $G_s\alpha$ protein activation are distinct.**

Heterotrimeric guanine nucleotide-binding proteins (G proteins)[1] couple heptahelical receptors to intracellular effectors and are composed of three subunits ($\alpha$, $\beta$, and $\gamma$) (reviewed in Refs. 1–3). The $\alpha$-subunits, which are distinct for each G protein, bind guanine nucleotide and modulate the activity of specific downstream effectors. For $G_s$, these include the stimulation of adenylyl cyclase and modulation of ion channels (4, 5). In the inactive state, GDP-bound $\alpha$-subunit is associated with a $\beta\gamma$-dimer. Upon receptor activation, the $\alpha$-subunit undergoes a conformational change resulting in the exchange of GTP for GDP and dissociation from $\beta\gamma$. While GTP is bound, the $\alpha$-subunit interacts with and regulates specific effectors. An intrinsic GTPase activity within the $\alpha$-subunit hydrolyzes bound GTP to GDP, returning the G protein to the inactive state. Analogs of GTP, such as GTP$\gamma$S and GDP-$AlF_4^-$, lock the G protein in the active state.

X-ray crystal structures reveal that G protein $\alpha$-subunits have two domains, a *ras*-like GTPase domain, which includes the regions for guanine nucleotide binding and effector interaction, and a helical domain, which may prevent release of GDP in the inactive state (6–12). Comparison of the crystal structures of inactive (GDP-bound) and activated (GTP$\gamma$S- or $AlF_4^-$-bound) $\alpha$-subunits demonstrates three regions (named switches 1, 2, and 3), the conformation of which changes upon switching from the inactive to active state. The movement of switches 1 and 2 is directly related to the presence of the $\gamma$-phosphate group, whereas switch 3 has no direct contact with bound guanine nucleotide. Upon activation, switches 2 and 3 move toward each other, and the two regions form multiple interactions that presumably stabilize the active state (7, 10). Switch 3 residues also make contacts with the helical domain that are important for high affinity guanine nucleotide binding (10, 15). At least for transducin, this region may also be important in effector activation (13).

We have previously shown that substitutions of the switch 3 residue Arg$^{258}$ impairs activation by receptor or $AlF_4^-$ (15).[2] The latter effect was the direct result of decreased GDP binding due to loss of contacts between the Arg$^{258}$ side chain and residues within the helical domain. The adjacent residue (Glu$^{259}$) is invariant in all known G protein $\alpha$-subunits and is predicted to be important in activation, because it makes interactions with switch 2 residues in the active state (7, 12). Moreover, this residue is mutated to a valine in a patient with Albright hereditary osteodystrophy (16). In the present report, we provide evidence that substitution of Glu$^{259}$ also leads to impaired activation, particularly by receptor or $AlF_4^-$. How-

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[1] The abbreviations used are: G protein, guanine nucleotide-binding protein; $G_s$, stimulatory G protein; $G_s\alpha$, $G_s$ $\alpha$-subunit; $G_s\alpha$-E259D, -E259A, -E259Q, and -E259V, $G_s\alpha$ mutant with Glu$^{259}$ substituted to aspartate, alanine, glutamine, and valine, respectively; $AlF_4^-$, mixture of 10 $\mu$M $AlCl_3$ and 10 mM NaF; GTP$\gamma$S, guanosine-5′-*O*-(3-thiotriphosphate); WT, wild type.

[2] All numbering is based on the $G_s\alpha$-1 sequence reported by Kozasa *et al*. (17).

TABLE I
*Adenylyl cyclase stimulation by $G_s\alpha$ mutants*

*In vitro* transcription/translation products were mixed with purified cyc- membranes and assayed for adenylyl cyclase stimulation as described under "Experimental Procedures." Results are expressed as the mean ± S.D. ($\sigma_n - 1$) of triplicate determinations and are corrected for the relative level of synthesis of each mutant to $G_s\alpha$-WT. $G_s\alpha$-E259V, -E259A, -E259Q, and -E259D were synthesized to 73, 78, 74, and 91% of $G_s\alpha$-WT levels, respectively, as determined by *in vitro* translation with [$^{35}$S]methionine, SDS-PAGE, and phosphorimaging. Background values determined from mock transcription/translation reactions (in pmol of cAMP/ml of translation medium/15 min: GTP, 29 ± 1; isoproterenol, 39 ± 5; GTPγS, 39 ± 2; and $AlF_4^-$, 64 ± 6) were subtracted from each determination.

| $G_s\alpha$ mutation | Isoproterenol (10 μM) | | | |
|---|---|---|---|---|
| | GTP (100 μM) | + GTP (100 μM) | GTPγS (100 μM) | $AlF_4^-$[a] |
| | *pmol of cAMP/ml of translation product/15 min (% of WT)* | | | |
| WT[b] | 20 ± 6 | 231 ± 6 | 167 ± 7 | 359 ± 41 |
| E259V | 3 ± 3 | 8 ± 7 (3 ± 3) | 18 ± 3 (11 ± 2) | 5 ± 5 (1 ± 1) |
| E259A | 8 ± 3 | 37 ± 9 (16 ± 4) | 65 ± 10 (39 ± 6) | 10 ± 10 (3 ± 3) |
| E259Q | 2 ± 1 | 26 ± 15 (11 ± 6) | 61 ± 5 (37 ± 3) | 12 ± 12 (3 ± 3) |
| E259D | 15 ± 6 | 160 ± 33 (69 ± 14) | 164 ± 6 (98 ± 6) | 118 ± 11 (33 ± 5) |

[a] 10 mM NaF, 10 μM $AlCl_3$, and 100 μM GDP.
[b] The results for $G_s\alpha$-WT are the same as previously published (15) because these were generated simultaneously with those obtained for the $G_s\alpha$-$Arg^{258}$ mutants.

ever, impaired activation of these mutants by $AlF_4^-$ is not the result of decreased GDP binding (as is the case for the $Arg^{258}$ mutants) but rather is the result of a decreased ability to bind the $AlF_4^-$ moiety. The crystal structure of GTPγS-bound $G_s\alpha$ reveals interactions between the acidic side chain of $Glu^{259}$ and basic residues within switch 2 that are important in maintaining the active state and in binding of $AlF_4^-$ (12). Although adjacent switch 3 residues in $G_s\alpha$ ($Arg^{258}$ and $Glu^{259}$) are both critical for activation, the mechanisms by which mutations of these residues result in defective activation are distinct.

## EXPERIMENTAL PROCEDURES

*Construction of $G_s\alpha$ Plasmids and in Vitro Transcription/Translation*—To generate $G_s\alpha$ $Glu^{259}$ mutants, polymerase chain reaction was performed as described previously (15) using linearized vector containing wild type $G_s\alpha$ cDNA as template. The upstream primer was 5′-GACAAAGTCAACTTCCACATGTTTGACGTGGGTGGCCAGCGCGATGAACG-3′, and the downstream mutagenic primers were as follows: 5′-GAGCCTCCTGCAGGCGGTTGGTCTGGTTGTCCACCCGGATGACCATGTTG-3′ for E259V, 5′-GAGCCTCCTGCAGGCGGTTGGTCTGGTTGTCCGCCCGGATGACCATGTTG-3′ for E259A, 5′-GAGCCTCCTGCAGGCGGTTGGTCTGGTTGTCCTGCCGGATGACCATGTTG-3′ for E259Q, and 5′-GAGCCTCCTGCAGGCGGTTGGTCTGGTTGTCGTCCCGGATGACCATGTTG-3′ for E259D. Each polymerase chain reaction product was digested with *Hin*cII and*Sse*8387I and ligated into the transcription vector pBluescript II SK (Stratagene, La Jolla, CA) that contained wild type human $G_s\alpha$ cDNA (splice variant $G_s\alpha$-1, Ref. 17) in which the same *Hin*cII-*Sse*8387I restriction fragment had been removed. Mutations were verified by DNA sequencing, and synthesis of full-length $G_s\alpha$ from each construct was confirmed by immune precipitation of *in vitro* translated products with RM antibody, directed against the carboxyl-terminal decapeptide of $G_s\alpha$ (18). *In vitro* transcription/translation was performed on $G_s\alpha$ plasmids as described previously (15, 19) using the TNT-coupled transcription/translation system from Promega, with the exception that in most experiments, no RNase inhibitor was added.

*Adenylyl Cyclase Assays*—Wild type and mutant $G_s\alpha$ *in vitro* transcription/translation products (10 μl of translation medium) were reconstituted into 25 μg of purified S49 cyc plasma membranes and tested for stimulation of adenylyl cyclase in the presence of various agents as indicated in Table I (15, 19, 20). Reactions were incubated for 15 min at 30 °C, and the amount of [$^{32}$P]cAMP produced was measured as described previously (21).

*Trypsin Protection Assays*—Limited trypsin digestion of *in vitro* translated $G_s\alpha$ was performed as described previously (15, 19). Briefly, 1 μl of *in vitro* translated [$^{35}$S]methionine-labeled $G_s\alpha$ was incubated in incubation buffer (20 mM HEPES, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol) with or without 100 μM GTPγS or 10 mM NaF/10 μM $AlCl_3$ at various temperatures for 1 h and then digested with 200 μg/ml tosyl-L-phenylalanine chloromethyl ketone-trypsin for 5 min at 20 °C. In some experiments, GDP was also included in the preincubation, and in other experiments the $MgCl_2$ concentration was varied. Reactions were terminated by boiling in Laemmli buffer. Digestion products were separated on 10% SDS-polyacrylamide gels, and the amount of 38-kDa protected fragment was measured by phosphorimaging. The percentage of protection is the signal in 38-kDa protected band divided by the signal in the undigested full-length $G_s\alpha$ band × 100.

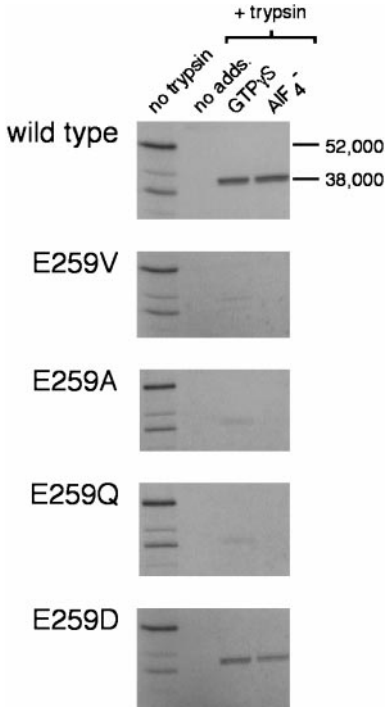


FIG. 1. **Trypsin protection of *in vitro* translated $G_s\alpha$-E259 mutants in the presence of GTPγS or $AlF_4^-$.** *In vitro* translates were digested with tosyl-L-phenylalanine chloromethyl ketone-trypsin (200 μg/ml) for 5 min at 20 °C after 1-h preincubations at 30 °C in the presence of GTPγS or $AlF_4^-$. For each $G_s\alpha$, the full-length undigested $G_s\alpha$ (52 kDa) is shown in the *far left lane* (*no trypsin*), and complete digestion in the absence of activators is demonstrated in the *second lane* (*no adds.*). The *two right lanes* show the amount of the 38-kDa protected band generated after trypsin digestion in the presense of either GTPγS (100 μM) or $AlF_4^-$. The smaller products in the *left lane* are due to initiation of protein synthesis at downstream methionine codons. Quantitation of trypsin protection assays for $G_s\alpha$-E259D is presented in Table II.

*Sucrose Density Gradient Centrifugation*—[$^{35}$S]Methionine-labeled $G_s\alpha$ was synthesized, and rate zonal centrifugation was performed on linear 5–20% sucrose gradients (200 μl) as described previously (19, 22). Gradients were prepared in 20 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol-PX. Six-μl fractions were obtained and analyzed by SDS-polyacrylamide gel electrophoresis, and the relative amount of $G_s\alpha$ in each fraction was quantified as described previously (19). To assess the ability of $G_s\alpha$ to bind to $G\beta\gamma$, *in vitro* translation products were preincubated for 1 h at 0 °C in the presence or absence of $G\beta\gamma$ (20 μg/ml) prior to centrifugation. In order to optimize separation between free α-subunit and heterotrimer,

0.15% (w/v) CHAPS was substituted for Lubrol-PX in the preincubations and gradients, and the samples were centrifuged at 120,000 rpm (627,000 × *g* at the maximum radial distance from the center of rotation ($R_{max}$) in a TLA-120.2 rotor (Beckman). G$\beta\gamma$ was isolated from bovine brain (23).

*Expression and Purification of $G_s\alpha$ from Escherichia coli*—Plasmid pQE60, containing the long form of bovine $G_s\alpha$ cDNA with a hexahistidine extension at the carboxyl terminus, was a generous gift of A. G. Gilman and R. K. Sunahara. The $Glu^{259}$ residue was mutated by site-directed mutagenesis using the Quickchange kit (Statagene). Each mutated cDNA was sequenced to confirm the presence of the desired mutation and to rule out polymerase chain reaction artifacts. After transformation into *E. coli* strain JM109, cultures were grown, $G_s\alpha$ expression was induced, and $G_s\alpha$ proteins were purified as described previously (15, 24), except that [GDP] was only 10 $\mu$M in the storage buffer.

*Guanine Nucleotide Binding Assays*—Assays measuring the rate of binding of GTP$\gamma$S were performed as described previously (15, 25). Briefly, 1–2 pmol of purified $G_s\alpha$ was incubated at 37 °C in a final volume of 2 ml containing 1 $\mu$M [$^{35}$S]GTP$\gamma$S (5,000–10,000 cpm/pmol) in 25 mM HEPES, pH 8.0, 1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.01% Lubrol-PX with or without 10 mM NaF/10 $\mu$M $AlCl_3$. At various times, 50-$\mu$l aliquots were removed and diluted with 2 ml of ice-cold stop solution (25 mM Tris-HCl, 100 mM NaCl, 25 mM $MgCl_2$, and 100 $\mu$M GTP) and maintained on ice until all samples were collected. Samples were then filtered under vacuum through nitrocellulose filters (Millipore) and washed twice with 10 ml of stop solution without GTP, and filters were dissolved in 10 ml of scintillation mixture. To determine the effect of $Mg^{2+}$ on the rate of GTP$\gamma$S dissociation, ~2.5 pmol of purified $G_s\alpha$ was loaded with [$^{35}$S]GTP$\gamma$S at 30 °C for 45 min in the presence of various free $Mg^{2+}$ concentrations. After addition of 100 $\mu$M cold GTP$\gamma$S, bound [$^{35}$S]GTP$\gamma$S was determined at various time points as described above. $k_{off}$ for GTP$\gamma$S dissociation was determined by fitting the data to the function $y = ae^{-kt} + b$ using the software GraphPad Prism, version 2.01. Free $Mg^{2+}$ concentrations were calculated as described (26).

TABLE II

*Effect of temperature and GDP on $AlF_4^-$-induced trypsin protection*

These data were obtained from experiments of the type presented in Fig. 1. The amount of the 38-kDa trypsin-stable $G_s\alpha$ fragment was determined by phosphorimaging, and for $G_s\alpha$-WT, it is expressed as a percent of undigested $G_s\alpha$ (mean ± S.E.). No protection was observed when $AlF_4^-$ and GTP$\gamma$S were excluded. Maximum trypsin protection has a theoretical limit of 71%, based on the removal of 2 of 7 total methionine residues by trypsin. For $G_s\alpha$-E259D, the data are expressed as percentage of wild type at each condition (mean ± S.E.). The number of experiments performed for each condition is shown in the right column.

| Temperature | Treatment | WT | E259D[a] | *n* |
|---|---|---|---|---|
| °C | | *% protection* | *% of wild type* | |
| 25 | $AlF_4^-$ | 56 ± 8 | 85 ± 15 | 4 |
| | $AlF_4^-$ + 2 mM GDP | 59 ± 6 | 60 ± 6 | 4 |
| 30 | $AlF_4^-$ | 59 ± 5 | 49 ± 7[b] | 8 |
| | $AlF_4^-$ + 2 mM GDP | 70 ± 10 | 36 ± 7[b] | 4 |
| | 100 $\mu$M GTP$\gamma$S | 60 ± 3 | 80 ± 5 | 4 |
| 37 | $AlF_4^-$ | 49 ± 5 | 7 ± 2[b,c] | 9 |
| | $AlF_4^-$ + 2 mM GDP | 63 ± 3 | 22 ± 2[b] | 4 |
| | 100 $\mu$M GTP$\gamma$S | 62 ± 5 | 72 ± 8 | 9 |

[a] The percentage of protection of $G_s\alpha$-E259D was significantly less than that of $G_s\alpha$-WT at all conditions except at 30 °C in the presence of $AlF_4^-$ (Student's *t* test).

[b] $p < 0.05$ *versus* GTP$\gamma$S (Student's *t* test).

[c] $p < 0.05$ *versus* $AlF_4^-$ + GDP (Student's *t* test).

RESULTS

*Substitution of $G_s\alpha$ $Glu^{259}$ Leads to Decreased Activation*—$G_s\alpha$ $Glu^{259}$ substitution mutants were cloned into the transcription vector pBluescript, and the *in vitro* transcription/translation products were compared with those of $G_s\alpha$-WT in various biochemical assays. We substituted $Glu^{259}$ with valine ($G_s\alpha$-E259V) because a mutation encoding this substitution has been identified in a patient with Albright hereditary osteodystrophy (16), a human disorder associated with heterozygous loss-of-function mutations of $G_s\alpha$ (27, 28). Because the presence of an amino acid with a bulky and branched side chain (valine) may introduce nonspecific steric effects, we also generated and analyzed additional mutants in which $Glu^{259}$ was replaced by alanine ($G_s\alpha$-E259A), glutamine ($G_s\alpha$-E259Q), or aspartate ($G_s\alpha$-E259D). In $G_s\alpha$-E259A, the acidic side chain was removed, whereas in $G_s\alpha$-E259Q it is converted to a residue in which the carboxyl group is replaced by a neutral amide group. In $G_s\alpha$-E259D, the charge of the residue at position $Glu^{259}$ is

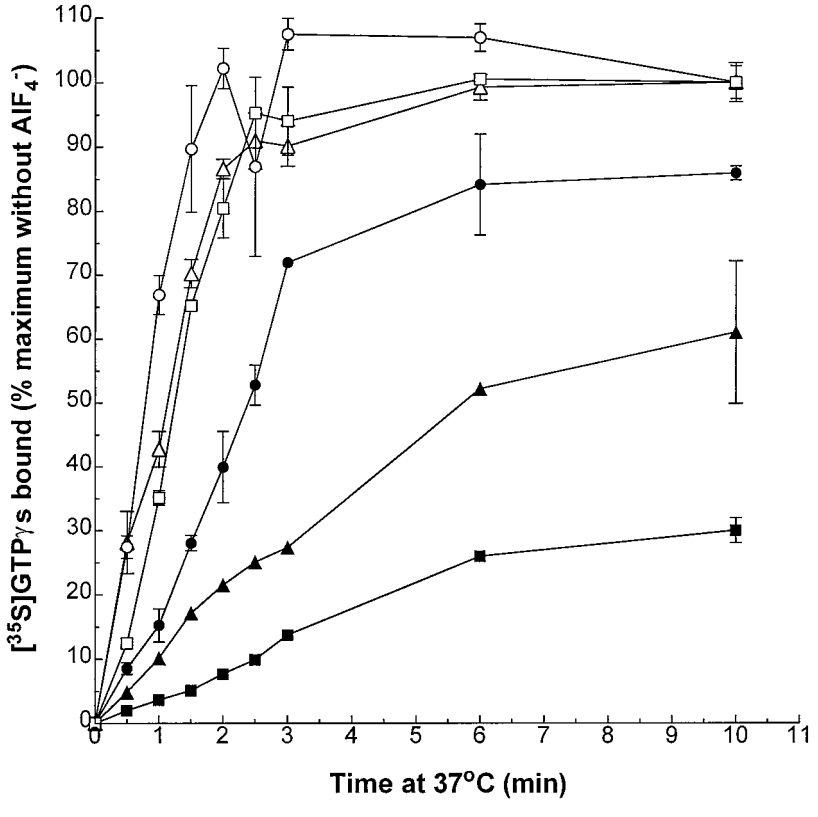


FIG. 2. **Time course of GTP$\gamma$S binding to purified $G_s\alpha$s in the presence or absence of $AlF_4^-$.** Bovine $G_s\alpha$-WT, -E259A, and -E259D, each with a carboxyl-terminal hexahistidine extension, were expressed and purified from *E. coli,* and the time course of GTP$\gamma$S binding for each was determined either in the presence (*filled symbols*) or absence (*open symbols*) of $AlF_4^-$. $G_s\alpha$-WT (■ and □), $G_s\alpha$-E259A (● and ○), and $G_s\alpha$-E259D (▲ and △) were incubated with 1 $\mu$M [$^{35}$S]GTP$\gamma$S (~10,000 cpm/pmol) at 37 °C for varying times, and the amount of bound GTP$\gamma$S was determined as described under "Experimental Procedures." For each $G_s\alpha$, each *data point* (with or without $AlF_4^-$) was normalized to maximal binding at 10 min in the absence of $AlF_4^-$. Each *data point* is the mean ± S.D. of triplicate determinations. This experiment was representative of three experiments. The $B_{max}$ values in the absence of $AlF_4^-$ were as follows: $G_s\alpha$-WT, 3 pmol; $G_s\alpha$-E259A, 2 pmol; and $G_s\alpha$-E259D, 1 pmol.

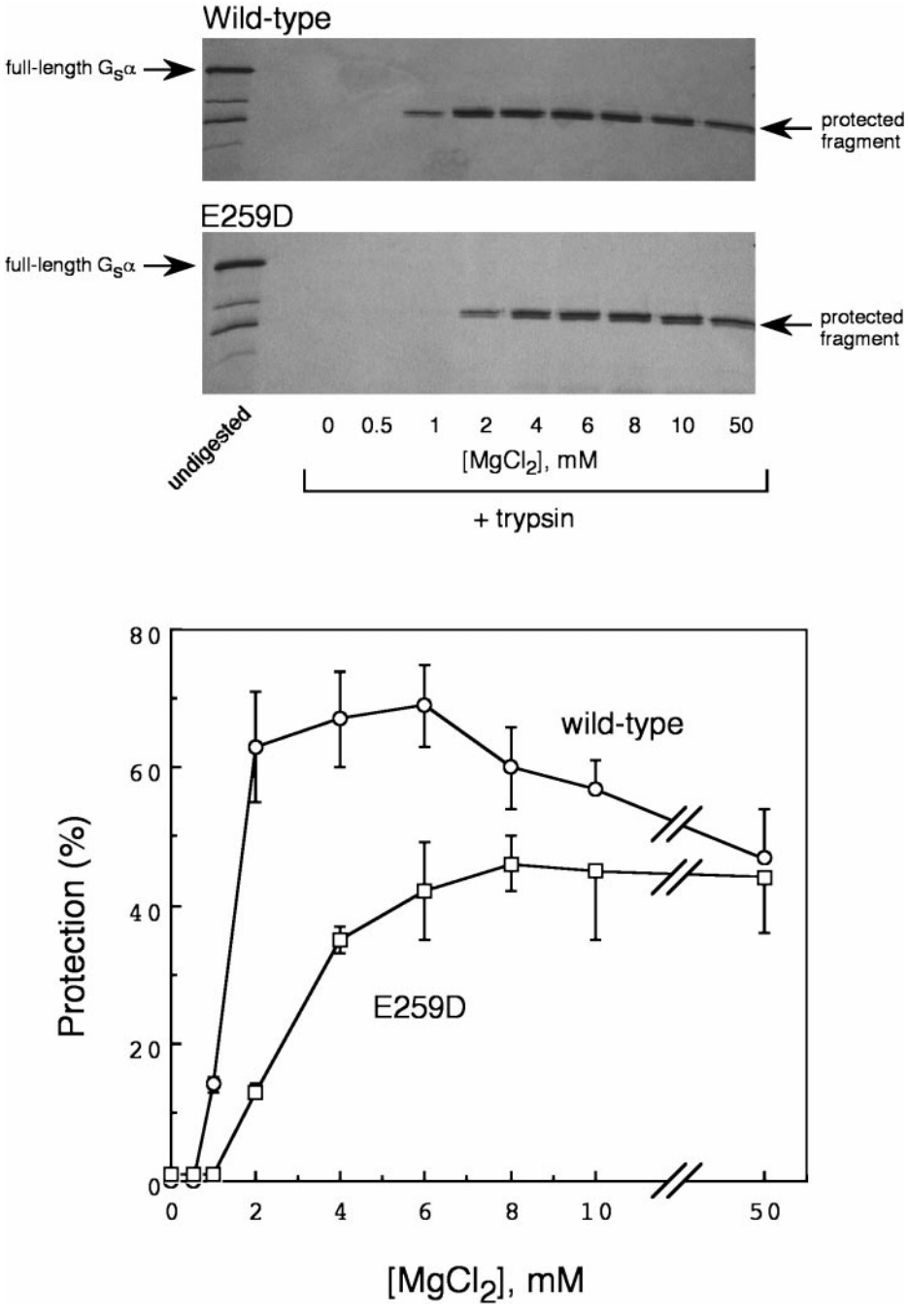


FIG. 3. **Effect of $MgCl_2$ concentration on trypsin protection of $G_s\alpha$-WT and -E259D in the presence of $AlF_4^-$.** Trypsin protection experiments were performed on $G_s\alpha$-WT and -E259D after incubation for 1 h at 30 °C in the presence of $AlF_4^-$ and various concentrations of $MgCl_2$ ranging from 0 to 50 mM. The *top two panels* show the gels from a representative experiment. The *bottom panel* shows the percentage of protection for $G_s\alpha$-WT and -E259D as a function of $MgCl_2$ concentration. Each *data point* represents the mean ± S.D. of three experiments.

maintained, but the length of the side chain is shortened by one methylene group.

After reconstitution of translation products into purified S49 cyc membranes (which lack endogenous $G_s\alpha$), $G_s\alpha$-E259V had markedly decreased ability to stimulate adenylyl cyclase in the presence of GTPγS, $AlF_4^-$, or activated receptor (isoproterenol + GTP) (Table I). For $G_s\alpha$-E259A and -E259Q, the ability to stimulate adenylyl cyclase was moderately reduced in the presence of GTPγS (~40% of $G_s\alpha$-WT) and more markedly reduced in the presence of $AlF_4^-$ or activated receptor. Stimulation of adenylyl cyclase by $G_s\alpha$-E259D was normal in the presence of GTPγS but moderately reduced in the presence of $AlF_4^-$ or activated receptor. Although the severity of the defect varied depending on which specific residue replaced $Glu^{259}$, for each $G_s\alpha$-$Glu^{259}$ mutant, GTPγS was the most effective activator and $AlF_4^-$ the least effective activator.

We next examined the ability of $AlF_4^-$ or GTPγS to protect each mutant from trypsin digestion, which measures the ability of each agent to bind to $G_s\alpha$ and induce the active conformation (29). In the inactive, GDP-bound state, two arginine residues within switch 2 (most likely $Arg^{228}$ and $Arg^{231}$, based upon sequence homology with transducin) are sensitive to trypsin digestion, leading to the generation of low molecular weight fragments. When $G_s\alpha$ attains the active conformation, these residues are inaccessible to trypsin digestion (7) and therefore trypsinization of activated $G_s\alpha$ generates a partially protected 38-kDa product. $G_s\alpha$-WT was well protected by $AlF_4^-$ or GTPγS at temperatures up to 37 °C (Fig. 1, Table II). At 30 °C, $G_s\alpha$-E259V, -E259A, and -E259Q showed little protection by GTPγS and no protection by $AlF_4^-$ (Fig. 1). In contrast, both GTPγS and $AlF_4^-$ were able to protect $G_s\alpha$-E259D, with GTPγS being a more efficient activator than $AlF_4^-$ (Fig. 1, Table II). Consistent with the results of the cyc reconstitution assays, $AlF_4^-$ was less effective than GTPγS in protecting all $G_s\alpha$-E259 mutants from

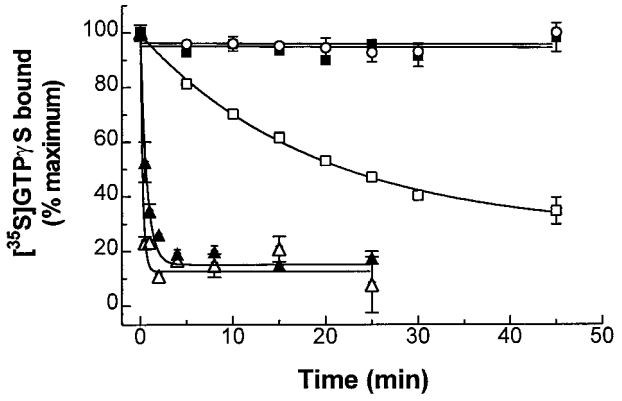


FIG. 4. **Effect of free $Mg^{2+}$ concentration on dissociation of GTPγS from purified $G_s\alpha$s.** Bovine $G_s\alpha$-WT and -E259D, each with a carboxyl-terminal hexahistidine extension, were expressed and purified from *E. coli,* and the time course of GTPγS dissociation was determined for each at various free $Mg^{2+}$ concentrations. $G_s\alpha$-WT (*closed symbols*) and $G_s\alpha$-E259D (*open symbols*) were preloaded with [$^{35}$S]GTPγS (5,000–10,000 cpm/pmol) at 30 °C for 45 min in the presence of 1 mM (○), 30 μM (■ and □), or no (▲ and △) free $Mg^{2+}$ (5 mM EDTA was added for the no $Mg^{2+}$ condition). After addition of 100 μM cold GTPγS, the amount of bound [$^{35}$S]GTPγS was determined at various time points. Each *data point* is the mean ± range of duplicate determinations. This experiment was representative of three experiments. Maximum [$^{35}$S]GTPγS in the presence of 5 mM EDTA was 0.5 pmol for $G_s\alpha$-WT and 0.3 pmol for $G_s\alpha$-E259D, and it was ~2.5 pmol for both in the presence of $Mg^{2+}$.

trypsin digestion.

Because the $G_s\alpha$-E259D encoded the most subtle structural change and had the smallest activation defect, we studied the ability of this mutant to be protected by GTPγS and $AlF_4^-$ at various temperatures and in the presence or absence of excess GDP (Table II). For $G_s\alpha$-R258 mutants, the activation defect in the presence of $AlF_4^-$ was more severe at higher temperatures and was reversible in the presence of excess GDP (15). Although raising the temperature had little effect on the ability of GTPγS to protect $G_s\alpha$-E259D from trypsin protection, temperature had a profound effect on protection by $AlF_4^-$, being 85, 49, and 7% of $G_s\alpha$-WT at 25, 30, and 37 °C, respectively. At 37 °C, addition of 2 mM GDP was able to somewhat reverse the defect in activation by $AlF_4^-$, although not to the extent that it was able to reverse the defect in the $G_s\alpha$-R258 mutants (15). Interestingly, addition of GDP lowered the ability of $AlF_4^-$ to protect $G_s\alpha$-E259D at 25 and 30 °C (Table II). Although this effect was consistently observed, we have no good explanation for this observation.

*Substitution of $G_s\alpha$ $Glu^{259}$ Has Little Effect on the Rate of GDP Release in the Basal State*—The impaired activation of $G_s\alpha$-$Glu^{259}$ mutants by $AlF_4^-$ could result from decreased affinity for $AlF_4^-$, decreased ability for the GDP-$AlF_4^-$ complex to activate the mutant $G_s\alpha$s, or decreased ability of the mutant $G_s\alpha$s to maintain the GDP-bound state because GDP binding is a prerequisite for $AlF_4^-$ binding and activation. For the $G_s\alpha$-$Arg^{258}$ mutants, impaired activation by $AlF_4^-$ is primarily the result of impaired GDP binding (15). The inability of GDP to significantly reverse the $AlF_4^-$-induced activation defect in $G_s\alpha$-E259D suggests that this defect is not due to defective GDP binding.

To directly evaluate the rate of GDP release in the basal state, we expressed and purified bovine $G_s\alpha$-WT, -E259A, and -E259D, each with a carboxyl-terminal hexahistidine tag, from *E. coli* and examined the time course of GTPγS binding. The rate of GTPγS binding has been shown to be limited by the rate of GDP dissociation, and the experimentally determined values of these two rates are essentially identical (30, 31). This assay has also been previously used as a measure of the GDP dissociation rate in other $G_s\alpha$ mutants(15, 32). Substitution of $Glu^{259}$ had little effect on the rate of GDP release in the basal state, as the time course of GTPγS binding at 37 °C (in the absence of $AlF_4^-$) is essentially identical for $G_s\alpha$-WT and -E259D, whereas the rate of GTPγS binding for $G_s\alpha$-E259A is only increased minimally (Fig. 2). Consistent with these results, the rate of increase of trypsin protection of $G_s\alpha$-WT and -E259D *in vitro* translation products in the presence of GTPγS was also identical (data not shown). These results demonstrate that unlike substitutions of $Arg^{258}$, the rate of GDP release is not significantly altered by substitution of $Glu^{259}$, and therefore the impaired activation by $AlF_4^-$ is not primarily due to decreased GDP binding.

*Substitution of $G_s\alpha$ $Glu^{259}$ Decreases $AlF_4^-$ Binding*—We next examined the ability of $AlF_4^-$ to interact with mutant $G_s\alpha$s in the GDP-bound state to determine whether the decreased activation of $G_s\alpha$-E259 mutants by $AlF_4^-$ is due to impaired $AlF_4^-$ binding. It has been shown previously that the rate and extent of GTPγS binding to G α-subunits is markedly reduced in the presence of $AlF_4^-$, presumably because the GDP-$AlF_4^-$ complex bound to Gα is more stable than GDP alone (8). Because $G_s\alpha$-WT, -E259D, and -E259A have similar rates of GTPγS binding in the absence of $AlF_4^-$, the time course of GTPγS binding in the presence of $AlF_4^-$ should reflect the ability of each form of $G_s\alpha$ to interact with $AlF_4^-$. Similar to previously reported observations (8), the rate and extent of GTPγS binding to $G_s\alpha$-WT was markedly reduced in the presence of $AlF_4^-$ (Fig. 2). In contrast, $AlF_4^-$ only partially reduced the rate and extent of GTPγS binding to $G_s\alpha$-E259D and had a minimal effect on the GTPγS binding curve for $G_s\alpha$-E259A (Fig. 2). These results are consistent with the results of adenylyl cyclase and trypsin protection assays, which demonstrate that $AlF_4^-$-induced activation is severely impaired in $G_s\alpha$-E259A but only partially impaired in $G_s\alpha$-E259D and suggest that the decreased ability of $AlF_4^-$ to activate $G_s\alpha$-E259 mutants is primarily due to decreased ability of the mutants to maintain $AlF_4^-$ in the guanine nucleotide binding pocket.

*Effect of $Mg^{2+}$ Concentration on Activation by $AlF_4^-$ and GTPγS Binding*—Substitution of $G_s\alpha$ $Arg^{231}$, a residue in switch 2 that interacts with switch 3 residues in the active state, leads to a defect in activation by $AlF_4^-$ that is more pronounced at low $Mg^{2+}$ concentrations (33). We therefore examined the effect of varying $Mg^{2+}$ concentration on the ability of $AlF_4^-$ to protect $G_s\alpha$-E259D from trypsin digestion. In the trypsin protection experiments shown in Fig. 1 and Table II, the $MgCl_2$ concentration was 10 mM (~9 mM free $Mg^{2+}$). Lowering the $MgCl_2$ concentration to 2 mM (~1 mM free $Mg^{2+}$) had no effect on the ability of $AlF_4^-$ to protect $G_s\alpha$-WT at 30 °C (Fig. 3). In contrast, lowering the $MgCl_2$ concentration below 8 mM (~7 mM free $Mg^{2+}$) further impaired the ability of $AlF_4^-$ to protect $G_s\alpha$-E259D in a concentration-dependent manner. Increasing the $MgCl_2$ concentration up to 100 mM did not reverse the defect at 37 °C (data not shown). These results are similar to those observed for the $G_s\alpha$-R231 mutant (33) and demonstrate that, like this mutant, the GDP-$AlF_4^-$-bound form of $G_s\alpha$-E259D has a lower apparent affinity for $Mg^{2+}$ than $G_s\alpha$-WT.

We next examined the effect of lowering the $Mg^{2+}$ concentration on the dissociation of GTPγS from $G_s\alpha$-E259D to determine whether or not the $Mg^{2+}$ dependence was specific for the GDP-$AlF_4^-$-bound form. The apparent $K_d$ of GTPγS-$G_s\alpha$-WT for $Mg^{2+}$ is very low (5–10 nM), and binding of GTPγS is essentially irreversible in the presence of micromolar concentrations of $Mg^{2+}$ (34). Consistent with previously published results (34), no dissociation of GTPγS from $G_s\alpha$-WT was observed at free $Mg^{2+}$ concentrations of 30 μM or higher (Fig. 4 and data not

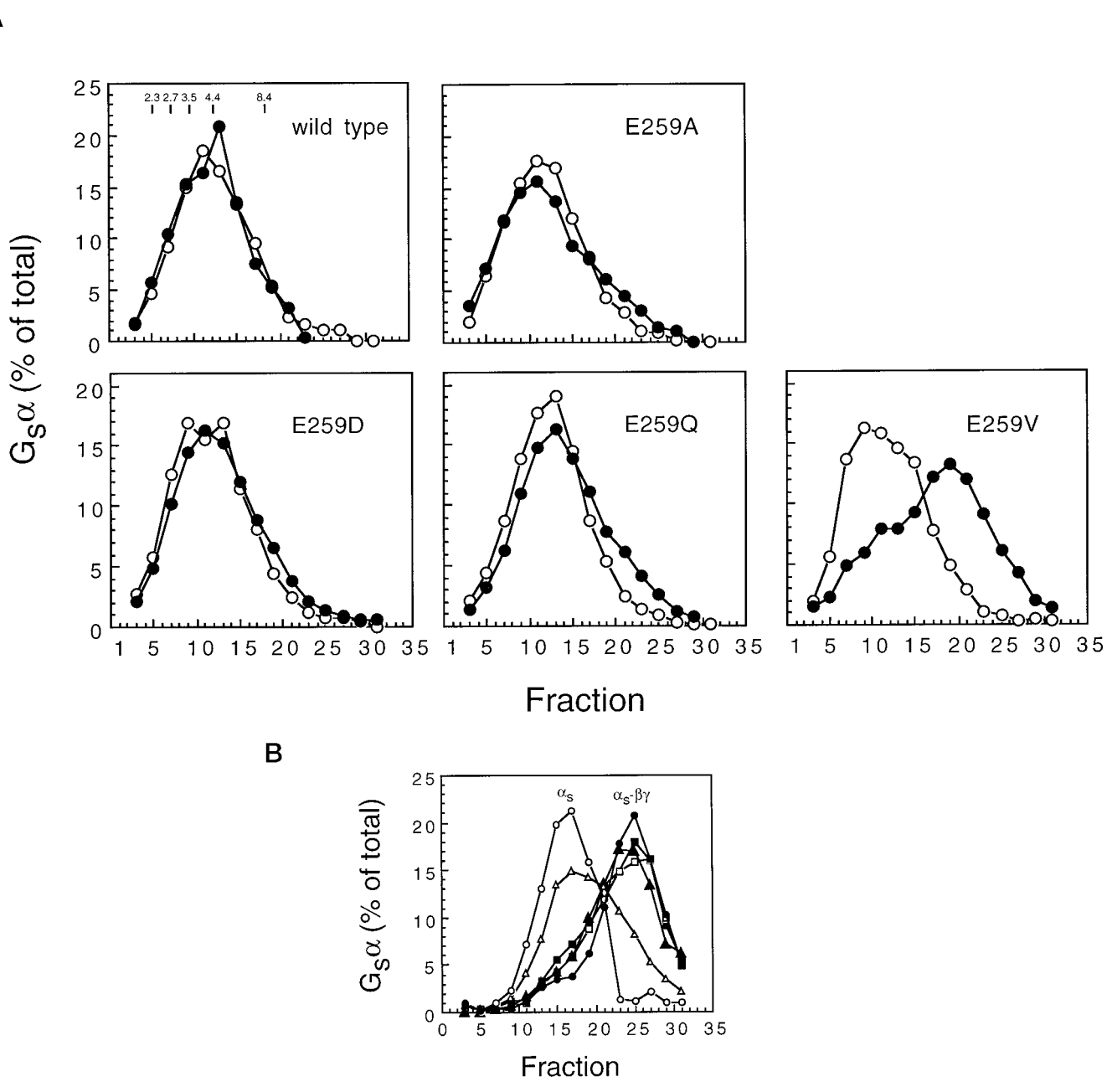


FIG. 5. **Sucrose density gradient centrifugation of $G_s\alpha$ *in vitro* translation products.** *A,* [$^{35}$S]methionine-labeled *in vitro* translates of both $G_s\alpha$-WT and -E259 mutants were preincubated for 1 h at 0 °C (○) or 30 °C (●) and subjected to sucrose density gradient centrifugation as described under "Experimental Procedures." Fractions (6 μl each) were collected, and odd-numbered fractions were analyzed by SDS-polyacrylamide gel electrophoresis and phosphorimaging (15, 19). The data are expressed as the percentage of total $G_s\alpha$ present in each fraction. Fraction 1 represents the top of the gradient. The position and S value of standard proteins are indicated at the *top* of the $G_s\alpha$-WT gradients. *B,* sucrose density gradient profiles of $G_s\alpha$-WT (●), $G_s\alpha$-E259A (■),$G_s\alpha$-E259Q (▲),$G_s\alpha$-E259D (□), and $G_s\alpha$-E259V (△) after preincubation for 1 h at 0 °C in the presence of purified bovine brain Gβγ (20 μg/ml). The profile for $G_s\alpha$-WT in the absence of Gβγ is also shown (○). All $G_s\alpha$-E259 (except $G_s\alpha$-E259V) mutants held at 0 °C in the absence of Gβγ had sucrose density gradient profiles similar to that of $G_s\alpha$-WT (data not shown). $G_s\alpha$-E259V had a somewhat broader peak at 0 °C that was unaltered in the presence of Gβγ. Conditions were modified to optimize separation between free α-subunit and heterotrimer as outlined under "Experimental Procedures." Similar results were obtained with the detergent octyl-β-glucoside (0.3% w/v).

shown), although GTPγS dissociated rapidly ($k_{off}$ = 2.5 min$^{-1}$) in the absence of $Mg^{2+}$ (5 mM EDTA). For $G_s\alpha$-E259D, GTPγS binding was essentially irreversible in the presence of 1 mM free $Mg^{2+}$, but in contrast to $G_s\alpha$-WT, GTPγS clearly dissociated from $G_s\alpha$-E259D in the presence of 30 μM free $Mg^{2+}$ (Fig. 4, $k_{off}$ = 0.05 min$^{-1}$). Dissociation of GTPγS from $G_s\alpha$-E259D ($k_{off}$ = 3.7 min$^{-1}$) was similar to that of $G_s\alpha$-WT in the absence of $Mg^{2+}$ (5 mM EDTA). Therefore, like GDP-$AlF_4^-$-$G_s\alpha$-E259D, GTPγS-$G_s\alpha$-E259D appears to have decreased affinity for $Mg^{2+}$, although the defects are apparent in the millimolar range for the former and micromolar range for the latter.

In contrast to $G_s\alpha$-E259D, there is a slow rate of dissociation of GTPγS from $G_s\alpha$-R231H in the presence of high $Mg^{2+}$ concentrations (33). Another $G_s\alpha$ mutant ($G_s\alpha$-G226A) also displays an abnormally high apparent $K_d$ for $Mg^{2+}$ to prevent GTPγS dissociation (34). Similar to $G_s\alpha$-E259D, GTPγS dissociates from $G_s\alpha$-G226A in the presence of micromolar concentrations of $Mg^{2+}$. There is also considerable dissociation of GTPγS from $G_s\alpha$-G226A even in the presence of maximal (millimolar) concentrations of $Mg^{2+}$, because this mutant cannot

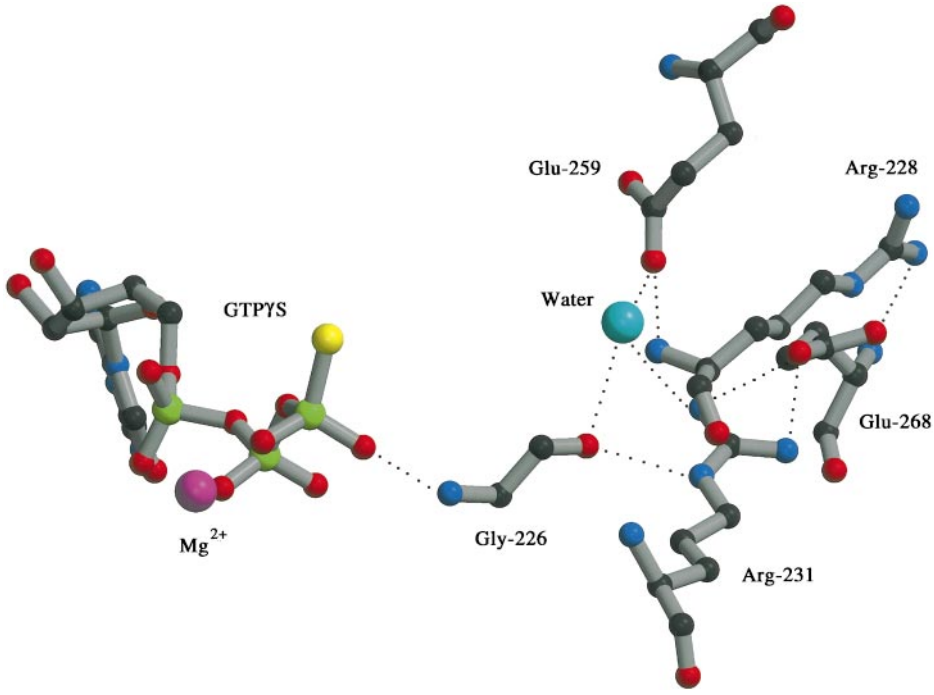


FIG. 6. **Crystal structure of $G_s\alpha$-GTPγS.** Detailed view of interactions between $Glu^{259}$ in switch 3, $Glu^{268}$ in α3, and residues in switch 2 and between $Gly^{226}$ and the γ phosphate of GTPγS. Hydrogen bonds are shown as *dotted lines*. The atom coloring scheme is as follows: *black,* carbon; *red,* oxygen; *blue,* nitrogen; and *yellow,* sulfur. $Mg^{2+}$ and water molecules are shown as *magenta* and *cyan* spheres, respectively. The figure was generated with MOLSCRIPT (39) and rendered with RASTER3D (37) using coordinates for the short form of bovine $G_s\alpha$-GTPγS (Protein Data Bank accession code 1AZT (12)), although the numbering on the figure corresponds to the long form of $G_s\alpha$ (17). This view is similar to that previously shown for transducin (6).

attain the active conformation that stabilizes the $Mg^{2+}$-GTPγS complex. The ability of $G_s\alpha$-E259D to irreversibly bind GTPγS in the presence of 1 mM $Mg^{2+}$ suggests that this mutant can attain the active conformation necessary to stabilize $Mg^{2+}$-GTPγS, consistent with the results obtained in the adenylyl cyclase and trypsin protection assays (Table I and Fig. 1).

*$G_s\alpha$-E259Q, E259A, and E259D, but not $G_s\alpha$-E259V, Maintain Normal Overall Conformation and Gβγ Interaction*—We examined the ability of each $G_s\alpha$-E259 mutant to interact with βγ by subjecting *in vitro* translates to sucrose density gradient centrifugation in the presence or absence of purified bovine brain βγ. We previously showed that $G_s\alpha$ has a sedimentation coefficient of ~3.7 S (15, 19). When *in vitro* translates of each $G_s\alpha$-E259 mutant was held on ice, the gradient profiles of all mutants were virtually the same as $G_s\alpha$-WT and consistent with the overall proper conformation (sedimentation coefficient, ~3.7 S) (Fig. 5*A*). When preincubated on ice with purified bovine brain βγ, $G_s\alpha$-WT, -E259Q, -E259A, and -E259D formed heterotrimers, as demonstrated by significant shifting of the peak toward the bottom of the gradient (Fig. 5*B*). In contrast, βγ had no effect on the sedimentation profile of $G_s\alpha$-E259V, indicating that this mutant does not interact with βγ. After preincubation at 30 °C, gradient profiles demonstrate that all mutants except $G_s\alpha$-E259V maintain an the normal 3.7 S conformation, whereas for $G_s\alpha$-E259V, the majority of the protein is a higher S value material and is presumably denatured (19). Therefore, the valine substitution probably alters the overall conformation and stability of the protein due to nonspecific steric effects of its bulky hydrophobic side chain. In contrast, the activation defect in $G_s\alpha$-E259A, E259Q, and E259D is not secondary to defects in thermostability or βγ binding.

## DISCUSSION

We previously reported that substitution of the $G_s\alpha$ switch 3 residue $Arg^{258}$ leads to impaired activation in the presence of $AlF_4^-$ or activated receptor (isoproterenol + GTP) but normal activation in the presence of GTPγS (15). The impaired activation by $AlF_4^-$ was reversible in the presence of excess GDP, and further characterization demonstrated a defect in GDP binding, presumably due to loss of direct contact between $Arg^{258}$ and a residue(s) in the helical domain that would open the cleft through which guanine nucleotide must exit. In this study, we examined the effect of substituting the adjacent switch 3 residue ($Glu^{259}$) on $G_s\alpha$ function for the following reasons: 1) this residue is strictly conserved among G protein α-subunits and therefore might have an important role in the biochemical function of these proteins; 2) upon activation, the $Glu^{259}$ side chain interacts with several residues in the switch 2 region (7, 12) and therefore substitutions of this residue might be predicted to directly impair G protein activation; 3) this $G_s\alpha$ residue is mutated to a valine in a patient with Albright hereditary osteodystrophy (16), a human disorder associated with heterozygous inactivating mutations within the $G_s\alpha$ gene (27, 28).

Substitution of $G_s\alpha$ $Glu^{259}$ to valine had a marked effect on the conformation and stability of the protein. This mutant was unable to interact with βγ, even though $Glu^{259}$ is not within the βγ interaction site (11). This mutant was also more thermolabile. Presumably, the presence of a bulky and branched side chain provided by valine introduces nonspecific steric effects that severely affect the conformation and stability of the protein. We would predict that the primary biochemical defect in the patient harboring this mutation is lack of expression of $G_s\alpha$-E259V in the membrane at physiological temperatures, similar to what is observed in other patients with mutants encoding unstable forms of $G_s\alpha$ protein (15, 19, 32).

In order to determine whether residue $Glu^{259}$ is critical in maintaining either the basal or activated state, we generated mutants with more subtle alterations of $Glu^{259}$ side chain. The most subtle mutation was $G_s\alpha$-E259D, in which the charge of the residue is maintained but the length of the side chain is shortened by one methylene group. We also made two mutants in which the side chain was either removed ($G_s\alpha$-E259A) or converted from an acidic to neutral amino acid ($G_s\alpha$-E259Q). In all three of these mutants, the overall conformation and stability, as well as the ability to interact with βγ, was maintained, as determined by sucrose density gradient experiments. Based upon adenylyl cyclase and trypsin protection assays, activation of $G_s\alpha$-E259D by GTPγS was normal, demonstrating that this mutant has not lost its intrinsic ability to attain the active conformation and activate adenylyl cyclase. However, this mutant had decreased ability of to be activated by $AlF_4^-$ or recep-

tor. $G_s\alpha$-E259Q and -E259A showed a more severe phenotype, with decreased activation in the presence of all agents. In all three mutants, GTPγS was the most efficient activator whereas $AlF_4^-$ was the least efficient. Mutation of the analogous residue in transducin ($Glu^{232}$) to leucine had no effect on the ability of the G protein to interact with βγ or its receptor (rhodopsin), but it did appear to decrease the ability of GTPγS to mediate trypsin protection and effector activation (13).

One possible mechanism for impaired activation by $AlF_4^-$ is decreased ability to maintain the GDP-bound state, because binding of GDP is a prerequisite for $AlF_4^-$ binding and activation. This is the primary mechanism by which substitutions of $G_s\alpha$ $Arg^{258}$ lead to impaired activation by $AlF_4^-$ (15). However, the ability of $G_s\alpha$-E259 mutants to maintain the GDP-bound state was similar to that of $G_s\alpha$-WT, as demonstrated by both $G_s\alpha$-E259A and -E259D having a rate of GDP release that was similar to $G_s\alpha$-WT, as well as an inability for excess GDP to significantly reverse the $AlF_4^-$-induced activation defect. Consistent with normal guanine nucleotide binding, both $G_s\alpha$-E259A and -E259D were thermostable. Binding of $AlF_4^-$ to the GDP-bound α-subunit results in formation of a stable and activated GDP-$AlF_4^-$-protein complex that mimics the transition state of the GTPase reaction and will slow the rate of GTPγS binding, probably by inhibiting GDP release (8). The ability of $AlF_4^-$ to inhibit the rate and extent of GTPγS binding to both $G_s\alpha$-E259A and -E259D was significantly reduced, suggesting that in these mutants the activation defect in response to $AlF_4^-$ is due at least in part to impaired $AlF_4^-$ binding. The fact that the activation defect is greater for $AlF_4^-$ than GTPγS suggests that mutation of $Glu^{259}$ has a more dramatic effect on stabilizing the transition ($AlF_4^-$-bound) state than the activated (GTPγS-bound) state.

It is of interest that the biochemical phenotype of our $G_s\alpha$-$Glu^{259}$ mutants is quite similar to that previously described for another $G_s\alpha$ mutant present in a patient with Albright hereditary osteodystrophy, in which the switch 2 residue $Arg^{231}$ is mutated to histidine ($G_s\alpha$-R231H) (33, 35). Similar to the $G_s\alpha$-$Glu^{259}$ mutants, this mutation leads to normal GTPγS-mediated but decreased $AlF_4^-$- and receptor-mediated activation. Moreover, similar to the $G_s\alpha$-R231H mutant, the $AlF_4^-$-induced activation defect in $G_s\alpha$-E259D was more pronounced at low $Mg^{2+}$ concentrations (33). This is not surprising, based upon mutual interactions between $Glu^{259}$ and $Arg^{231}$ present in the active (GTPγS-bound) conformation of $G_s\alpha$ (Fig. 6). Upon activation, interactions between switches 2 and 3 stabilize the GTP-bound form of the G protein. Specifically, $Arg^{231}$ in switch 2 interacts with $Glu^{259}$ in switch 3 through a water molecule and directly with $Glu^{268}$ in the α3 helix (Fig. 6). Conversely, $Glu^{259}$ interacts with two basic switch 2 residues, $Arg^{228}$ and $Arg^{231}$. Both $Arg^{231}$ and $Glu^{259}$ interact with $Gly^{226}$, a residue that is critical for both $AlF_4^-$ binding (36) and conformational switching of switch 2 upon binding of GTP or $AlF_4^-$ (29). Therefore, the impaired activation and $AlF_4^-$ binding observed in $G_s\alpha$-$Glu^{259}$ mutants might be the direct result of loss of contacts with $Gly^{226}$. Loss of these contacts may also result in the apparent decreased affinity of $G_s\alpha$-$Glu^{259}$ and R231H mutants for $Mg^{2+}$ because mutation of $Gly^{226}$ to alanine also lowers the apparent affinity of $G_s\alpha$ for $Mg^{2+}$ (34).

Mutation of $Glu^{259}$ leads to a subtle defect in receptor-mediated activation (at least when compared with activation by GTPγS). $G_s\alpha$-E259 mutants are able to bind βγ, and mutation of the analogous residue in transducin ($Glu^{232}$) has no effect on interactions with βγ or receptor (13). It has been proposed that decreased receptor activation of $G_s\alpha$-R231H is due to a conditional defect in GTP binding, which is more pronounced in states in which guanine nucleotide binding is destabilized (such as interaction with activated receptor (33)). Our results are consistent with those observed with $G_s\alpha$-R231H and support this hypothesis.

In conclusion, this study provides further evidence for the role of switch 3 in the activation mechanism and demonstrates the importance of interactions between $Glu^{259}$ and switch 2 residues. Taken together with the prior studies on $Arg^{258}$ mutants (15, 38), the present results demonstrate the importance of switch 3 in maintaining both the basal and active states.

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